The Isolation and Amino Acid Composition of Two Peptides from Chymotryptic Digests of β-Lactoglobulins A and B

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Short-term chymotryptic digests have been carried out with β-lactoglobulins A and B, performic acid-oxidized and S-sulfonated derivatives. Differences in the peptide patterns have been found after high-voltage electrophoresis only when disulfide bonds were previously cleaved. Two peptides have been isolated from chymotryptic digests of S-sulfonated β-lactoglobulins, and their amino acid compositions have been determined to be as follows: For the peptide from the β-A derivative: Asp_2, Thr_1, Glu_4, Pro_1, Ala_3, Val_1, $Ileu_2$, Phe_1, ½ Cys_1, Lys_4; and for the peptide from β-B: Asp_1, Thr_1, Glu_4, Pro_1, Ala_3, Val_1, $Ileu_2$, Phe_1, ½ Cys_1, Lys_4, Gly_1. The sole difference lies in the aspartic acid–glycine content. The valine–alanine difference in β-lactoglobulins A and B remains unaccounted for in the present study.

INTRODUCTION

Aschaffenburg and Drewry (1) demonstrated that two genetically different β-lactoglobulins exist and that these electrophoretically distinguishable proteins, called β-lactoglobulin A and β-lactoglobulin B (2), are produced exclusively by certain individual cows. End-group analysis (3–8) in combination with physicochemical criteria (9, 10) show that each genetic species of mol. wt. 35,500 consists of two polypeptide chains of approximately half this weight. The electrophoretic difference has been shown by Tanford and Nozaki (11) to be due to the presence in β-A[2] of two carboxyl groups per molecule more than the number titratable in β-B. The chemical differences between the two β-lactoglobulins have been found by Gordon *et al.* (12, 13) and independently by Piez *et al.* (14, 15) to be in the content of four amino acids: β-A having a higher aspartic acid and valine content, while β-B contains more glycine and alanine. The present report deals with the isolation of peptides from chymotryptic digests of S-sulfonated β-A and β-B and the similarity of the peptide patterns save for a single pair, in which one β-A aspartic acid residue is replaced by one glycine residue in the corresponding β-B peptide. These results confirm, in part, the total amino acid analysis of the genetically different proteins and strongly suggest that the β-A - β-B system represents, like hemoglobin (16), genetic substitutions of single amino acid residues in otherwise identical polypeptide chains.

[1] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[2] Abbreviations: β-A, β-lactoglobulin A; β-B, β-lactoglobulin B; S-β-A, S-sulfo-β-lactoglobulin A; S-β-B, S sulfo-β-lactoglobulin B.

EXPERIMENTAL

MATERIALS

β-A and β-B were prepared according to the method of Aschaffenburg and Drewry (1) from the milk of a number of typed cows which was made available by Drs. N. D. Bayley and C. A. Kiddy, Dairy Cattle Research Branch, U. S. Department of Agriculture, Beltsville, Maryland. Each protein was recrystallized three times and either lyophilized or stored as a slurry under distilled water saturated with toluene. Performic

acid-oxidized β-lactoglobulins were prepared by the method of Hirs (17) and S-sulfonated derivatives by a modification (18) of the Swan cupric ion–sulfite reaction (19). Chymotrypsin was a crystalline preparation obtained from Armour Biochemical,[3] and all reagents employed in connection with the high-voltage paper electrophoresis were analytical grade.

Chymotryptic Digestions

Digestions were carried out under nitrogen in 4-ml. volumes, using unbuffered systems and maintaining pH at 8.0 by automatic addition of 0.1 *N* NaOH in a pH-stat. For preliminary runs, 100-mg. samples of modified or native proteins were digested at 37°C. with an enzyme–substrate weight ratio of 1:100. The reaction was adjusted to pH 6.5 with 0.1 *N* HCl at indicated times. The small amounts of gelatinous precipitate which formed at this time were centrifuged off. Aliquots of the clear supernatant, containing 1–2 mg. protein, were dried in a vacuum desiccator and analyzed by high-voltage paper electrophoresis on Whatman 3MM paper, using a horizontal water-cooled plate, and the buffers and techniques of Ingram (20). Analytical electrophoretic runs were generally carried out at pH 6.4 for 2 hr. with a potential of 45 v./cm. The papers were then air-dried and peptides were visualized by dipping the strips into 0.2% ninhydrin in acetone, drying again, and heating 2 min. in a 110° oven. Preparative electrophoretic runs were made under the same conditions, using aliquots of digests corresponding to approximately 20 mg. protein and, after drying, a guide strip was cut from the edge and the band or bands of interest located by ninhydrin. The section of paper involved was then cut out and the peptide eluted with 1.0 *M* acetic acid. The eluates of each peptide were then combined and taken to dryness *in vacuo* over P_2O_5 and soda–lime. The crude peptides were purified by repeating the paper electrophoresis first with a pyridine–acetic acid buffer, pH 3.6, (3000 v., and 90 min.) and finally with the pyridine–acetic acid buffer of pH 6.4 (3500 v., 120 min.). The purified peptides were dissolved in 0.1 *M* acetic acid to a final volume of 10 ml.

Amino Acid Analysis

The amino acid composition was determined as previously described (12, 13), according to the procedure of Spackman *et al.* (21) with a Phoenix model K-5000 Amino Acid Analyzer.[3] The tryptophan content was determined by the method of Spies and Chambers (22).

[3] It is not implied that the U. S. Department of Agriculture recommends the above company to the possible exclusion of others in the same business.

RESULTS AND DISCUSSION

Chymotryptic Digests

Figure 1 is a record of the base uptake during chymotryptic digestion of the proteins and various derivatives. The amount of base utilized levels off in relatively short periods of time, and even after addition of more enzyme in the cases indicated there is no appreciable increase in the rate of digestion. Only in the case of the preparative digestion was there still any appreciable base uptake when the reaction was terminated. A correlation between the amount of base utilized and the number of peptide bonds hydrolyzed was not determined, since too many assumptions concerning the specificity of chymotrypsin and the p*K* of the groups titrated are required. In addition, it was felt that this information was not pertinent to the present study. The figure includes only the reactions involving β-A and its derivatives since the data obtained with β-B were essentially the same.

Figure 2 is a photograph of the pH 6.4 ionogram of the chymotryptic digests of performic acid-oxidized (P), S-sulfonated (S), and native (N) β-lactoglobulins. For each strip the digest of the β-B protein is shown in the lower half with the negative pole toward the left. The native proteins revealed no apparent differences in their peptide patterns under the conditions used. This was true even after two-dimensional chromatograms were obtained (20). However, the pattern of the performic acid-oxidized material clearly showed a peptide from the β-B protein migrating toward the negative pole and absent in the A peptide pattern, while the β-A pattern revealed a peptide located near the point of application (thin pencil line) and absent in the β-B pattern. These same peptides are visible in the patterns given by the S-sulfonated derivatives. In addition, there appeared to be differences in peptides migrating toward the positive pole in the S-sulfonated peptide pattern. However, when these latter peptides were isolated and analyzed, their amino acid compositions did not appear to

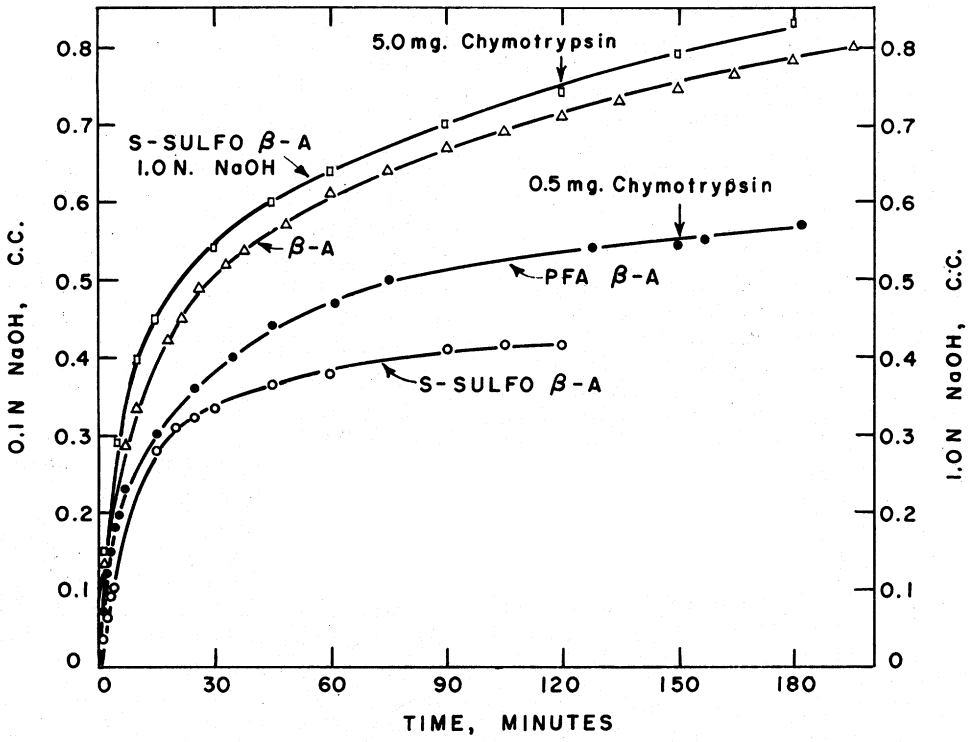

FIG. 1. Base uptake during chymotryptic digest of △——△ native β-A, ●——● performic acid oxidized β-A, ○——○ S-sulfonated β-A (pH 8.0, t = 37°C., enzyme–substrate ratio 1:100) □——□ S-sulfonated β-A (preparative digest pH 8.0, t = 25°C. Initial enzyme–substrate ratio 1:100).

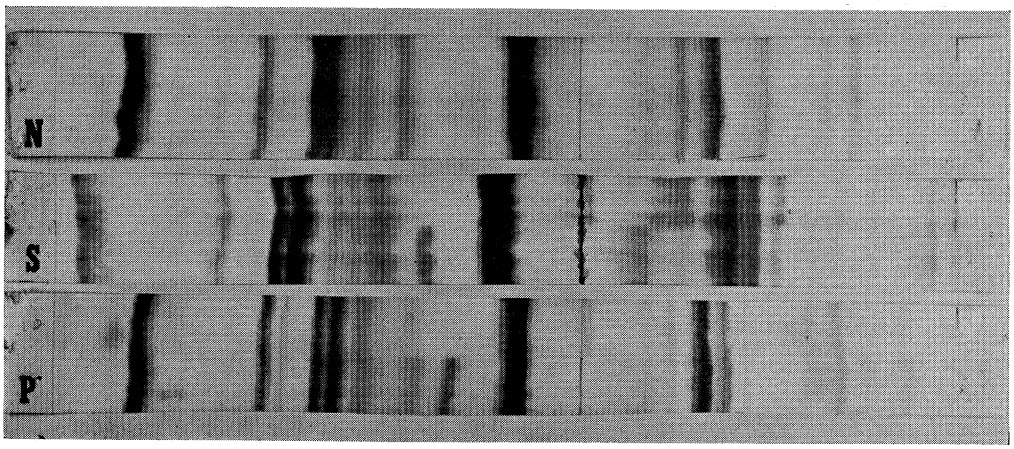

FIG. 2. High voltage electrophoresis diagram of chymotryptic digests of performic acid oxidized (P), S-sulfonated (S), and native (N) β-lactoglobulins. For each strip, digest of β-B protein in lower half with negative pole toward the left. Electrophoresis at pH 6.4 for 2 hr. at 45 v./cm.

be related. In contrast to this, the analyses of the peptide migrating to the negative pole (B peptide) and the peptide remaining near the point of application (A peptide) showed that these peptides were composed of essentially the same kind and amounts of amino acids except glycine and aspartic acid. This will be further discussed below. In passing, it should be noted that there appears to be a requirement for disulfide-bond cleavage before electrophoretic peptide differences are obtainable in this particular case.

Preparative Digest and Isolation of Two Peptides

The use of the S-sulfonated proteins for a large-scale preparative run was decided upon because the preliminary experiments revealed more peptide differences in the case of the S-sulfonated derivatives (see Fig. 2). Preparative digests using 2.0 g. of S-sulfonated β-lactoglobulins were carried out in 80-ml. volumes under the same conditions as above except that the temperature was 25°C. and the reaction time was 3 hr.

Figure 3 is the peptide pattern given by

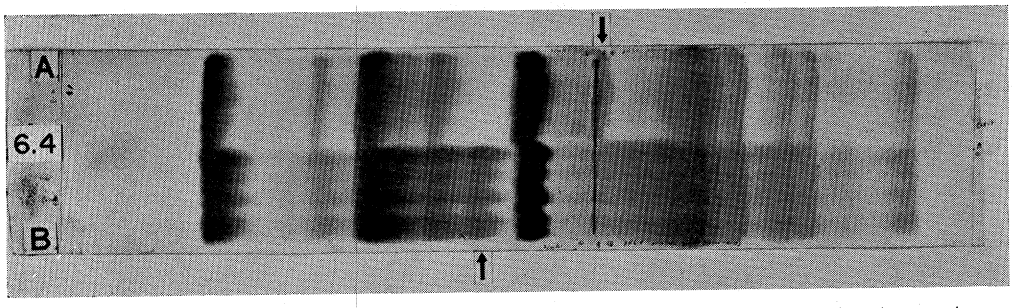

Fig. 3. Peptide pattern of chymotryptic digest of S-sulfonated β-lactoglobulins A = β-A, B = β-B electrophoresis at pH 6.4 for 2 hr. at 45 v./cm. Arrows indicate differing peptides isolated.

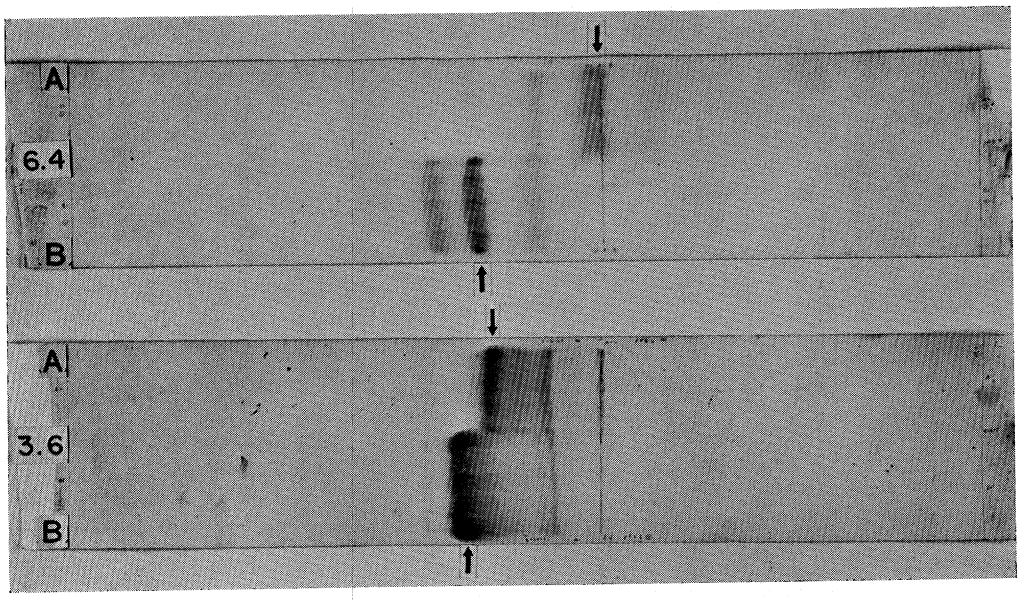

Fig. 4. Peptides A and B after electrophoresis at pH 3.6 examined at pH's 6.4 and 3.6.

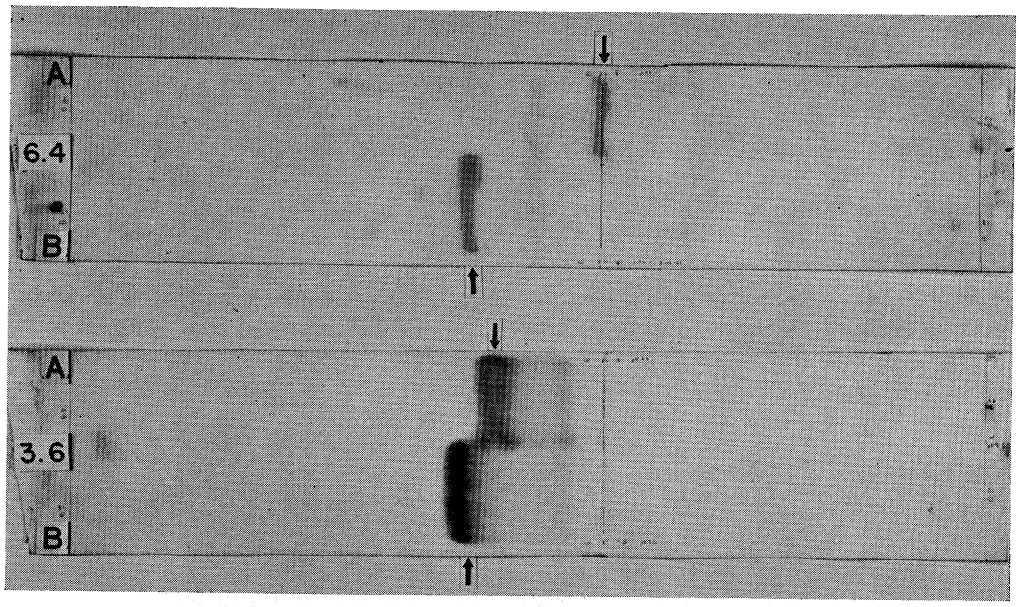

FIG. 5. Peptides A and B after final electrophoretic purification at pH 6.4 examined at pH's 6.4 and 3.6.

TABLE I

COMPOSITION OF PEPTIDES

Amino acids	A Peptide				B Peptide			
	μmoles[a] found	μmoles found / μmoles glutamic acid	X4	Residues	μmoles found	μmoles found / μmoles glutamic acid	X4	Residues
Aspartic acid	.28	.54	2.16	2	.24	.30	1.20	1
Threonine	.13	.25	1.00	1	.20	.25	1.00	1
Serine	.015	.03	.12	0	.014	.02	.08	0
Glutamic acid	.52	1.00	4.00	4	.80	1.00	4.00	4
Proline	.12	.23	.92	1	.17	.21	.84	1
Glycine	.023	.04	.16	0	.22	.28	1.12	1
Alanine	.39	.75	3.00	3	.56	.70	2.80	3
½ Cystine	.11	.21	.84	1	.15	.19	.76	1
Valine	.14	.27	1.08	1	.21	.26	1.04	1
Leucine	.031	.06	.24	0	.11	.14	.56	0[b]
Isoleucine	.29	.56	2.24	2	.41	.51	2.04	2
Tyrosine	—	—	—	—	.021	.03	.12	0
Phenylalanine	.11	.21	.84	1	.16	.20	.80	1
Lysine	.49	.94	3.76	4	.89	1.11	4.44	4
Tryptophan	—	—	—	—	.06	.075	.30	0

[a] All values obtained by column chromatography except tryptophan by the method of Spies and Chambers (22).

[b] See text for discussion of leucine value.

these digests after 2 hr. electrophoresis at pH 6.4. It is similar to the one obtained in the smaller scale experiments except that the differences in anodic peptides are less apparent. The peptides of interest are indicated by the arrows. These were isolated as described previously and then purified by successive electrophoresis and elution, first at pH 3.6, and finally again at pH 6.4. The analytical patterns at two pH's after the preparative electrophoresis at pH 3.6, and patterns after the final purification at 6.4 are shown in Figs. 4 and 5, respectively. The total amount of peptide obtained was 2.4 μmoles for the A peptide and about 4 μmoles for the B.

Results of amino acid analysis of the peptides are presented in Table I. The only major differences in composition are in aspartic acid and glycine content, the A peptide containing one more residue of aspartic acid and one less of glycine than the B peptide. The presence of about half a residue of leucine and a small excess of lysine in the B peptide are probably the result of impurities still present after the process of purification. This view is supported by the presence of detectable amounts of tyrosine and tryptophan in the B peptide, and the absence of these trace amino acids in the A peptide. The evidence is certainly presumptive that we are indeed dealing with the aspartic acid–glycine substitution responsible for the difference in the titration curve data of Tanford and Nozaki (11) and accounting for the aspartic acid–glycine difference in amino acid composition already reported (12–15).

The absence of tryptophan in these chymotryptic peptides is in contrast to its occurrence in two tryptic peptides also revealing the aspartic acid–glycine substitution.[4, 5] The chymotryptic peptides showing the valine–alanine difference have not yet been located and further work toward this end is in progress.

[4] R. Townend, W. G. Gordon, and V. M. Ingram, unpublished results.

[5] K. A. Piez, E. W. Davie, J. E. Folk, and J. A. Gladner, private communication.

REFERENCES

1. Aschaffenburg, R., and Drewry, J., *Nature* **176,** 218 (1955).
2. Aschaffenburg, R., and Drewry, J., *Nature* **180,** 376 (1957).
3. Fraenkel-Conrat, H., *J. Cellular Comp. Physiol.* **47,** *Suppl.* 1, 133 (1956).
4. Niu, C. I., and Fraenkel-Conrat, H., *J. Am. Chem. Soc.* **77,** 5882 (1955).
5. Neurath, H., Gladner, J. A., and Davie, E. W., *in* "Symposium on the Mechanism of Enzyme Action" (W. D. McElroy, and B. Glass, eds.), p. 50. Johns Hopkins Univ. Press, Baltimore, 1954.
6. Davie, E. W., Newman, C. R., and Wilcox, P. E., *J. Biol. Chem.* **234,** 2635 (1959).
7. Kalan, E. B., and Greenberg, R., *Federation Proc.* **20,** 384 (1961).
8. Kalan, E. B., and Greenberg, R., *Arch. Biochem. Biophys.* **95,** 79 (1961).
9. Timasheff, S. N., and Townend, R., *J. Am. Chem. Soc.* **83,** 470 (1961).
10. Green, D. W., and Aschaffenburg, R., *J. Mol. Biol.* **1,** 54 (1959).
11. Tanford, C., and Nozaki, Y., *J. Biol. Chem.* **234,** 2874 (1959).
12. Gordon, W. G., Basch, J. J., and Kalan, E. B., *Biochem. Biophys. Research Communs.* **3,** 672 (1960).
13. Gordon, W. G., Basch, J. J., and Kalan, E. B., *J. Biol. Chem.* **236,** 2908 (1961).
14. Piez, K. A., Davie, E., Folk, J. E., and Gladner, J. A., *Federation Proc.* **20,** 384 (1961).
15. Piez, K. A., Davie, E. W., Folk, J. E., and Gladner, J. A., *J. Biol. Chem.* **236,** 2912 (1961).
16. Ingram, V. M., *Biochim. et Biophys. Acta* **36,** 402 (1959).
17. Hirs, C. H. W., *J. Biol. Chem.* **219,** 611 (1956).
18. Weil, L., and Seibles, T. S., *Arch. Biochem. Biophys.* **84,** 244 (1959).
19. Swan, J. M., *Nature* **180,** 643 (1957).
20. Ingram, V. M., *Biochim. et Biophys. Acta* **28,** 539 (1958).
21. Spackman, D. H., Stein, W. H., and Moore, S., *Anal. Chem.* **30,** 1190 (1958).
22. Spies, J. R., and Chambers, D. C., *Anal. Chem.* **20,** 30 (1948).